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Effects of Stocking Density on Intestinal Health of Juvenile *Micropterus salmoides* in Industrial Aquaponics

Jishu Zheng [1,†], Zhengxi Wang [1,2,†], Decheng Pu [1,*], Peiyuan Li [1], Xiuli Wei [1], Mai Li [1], Dongsheng Li [1], Lihong Gao [1] and Xuliang Zhai [3]

[1] Key Laboratory of Smart Agricultural Technology in the Southwest Mountains, Ministry of Agriculture and Rural Affairs (Co-Construction by Ministry and Province), Chongqing Academy of Agricultural Sciences, Chongqing 400715, China; imzhengjishu@163.com (J.Z.); 13036328893@163.com (Z.W.); lpy0616@126.com (P.L.); wei77703863@126.com (X.W.); rlm410@163.com (M.L.); dawson.lee@foxmail.com (D.L.); grantsan@163.com (L.G.)

[2] Research Center of Fishery Resources and Environment, Conservation and Research Center for Aquatic Biodiversity in the Upper Reaches of Yangtze River Ministry of Agriculture and Rural Affairs, College of Fisheries, Southwest University, Chongqing 400715, China

[3] Chongqing Fisheries Technical Extension Center, Chongqing 400715, China; zhaizige@sohu.com

* Correspondence: pudc801@sina.com

† These authors contributed equally to this work.

Abstract: Stocking density is a key factor affecting the health of aquatic organisms in industrial aquaponics. In this study, *Micropterus salmoides* were assigned to one of two density groups (160 and 120 fish/m^3) for 40 days. The growth performance, intestinal morphology, enzyme activity, and microbial communities were compared between the two density groups. The findings revealed that the higher stocking density condition exhibited an increased weight gain rate and specific growth rate during the developmental phase of the juvenile *M. salmoides*. Moreover, remarkable increases in villi height, villi width, and muscular layer thickness were observed. Additionally, this elevated stocking density condition also enhanced the activity of intestinal antioxidant enzymes, consequently improving the structural integrity of the intestine and augmenting the digestive and absorptive capacities of the juvenile *M. salmoides*. With regard to the intestinal microbial community, the dominant phyla detected were Firmicutes and Proteobacteria. However, under the higher stocking density condition, there was a significant upsurge in the abundance of *Mycoplasma*. Consequently, it is advised to mitigate the abundance of such pathogenic microorganisms through the regulation of the water environment during the aquacultural process. Drawing from recent investigations on the impact of various factors on the intestinal microbiota of *M. salmoides*, it can be deduced that the composition of the intestinal microbiota is closely intertwined with factors including aquaculture practices, feed composition, water environment, and developmental stage. In summary, the aforementioned research findings possess noteworthy implications for the control of stocking density in the cultivation of juvenile *M. salmoides*.

Keywords: enzyme activities; growth performance; intestinal morphology; intestinal microbial community

Key Contribution: This study shows that a stocking density of 160 fish/m^3 can promote the growth and intestinal structure of juvenile *Micropterus salmoides*, thereby improving their digestive and absorption capacity. In addition, a significant increase in *Mycoplasma* abundance may lead to disease outbreaks. Drawing from recent investigations of the impact of various factors on the intestinal microbiota of *M. salmoides*, it can be deduced that the composition of the intestinal microbiota is closely intertwined with factors including aquaculture practices, feed composition, water environment, and developmental stage.

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1. Introduction

The maintenance of optimal intestinal health is crucial for promoting fish growth and immune functionality, as any perturbation to intestinal health can have a direct impact on their productive performance [1]. Currently, the assessment of intestinal tissue morphology [2], intestinal enzyme activity [3], and the structure and functionality of microbial communities [4] can provide valuable insights into fish intestinal health. Serving as a vital immune organ, the fish intestine represents the largest and most intricate secretory organ and tissue, playing a pivotal role in nutrient digestion and absorption within the piscine organism [5,6]. Furthermore, it serves as a biological, physical, and chemical barrier, safeguarding against bacterial infiltration and the uptake of toxic substances [7,8]. Consequently, intestinal morphology and health status unavoidably exert a profound influence on critical physiological processes in fish, including growth, development, and reproduction [9]. Previous studies have unveiled that different stocking densities can elicit distinct effects on the height of columnar cells, thickness of the muscular layer, and dimensions of goblet cells in the anterior, middle, and posterior segments of *Clarias leather* intestines [10], hence exerting an impact on intestinal health.

The digestive enzymes present in fish facilitate the breakdown of macromolecules into smaller, bioavailable compounds, thereby augmenting their nutrient absorption capacity and growth performance [11]. Consequently, the assessment of intestinal digestive enzyme activity has emerged as a pivotal determinant influencing organismal survival and growth [12]. Furthermore, during cellular stress, mitochondria exhibit heightened production of reactive oxygen species. To counterbalance this oxidative burden, the body's antioxidant system orchestrates an upregulation in the activity of antioxidant enzymes and antioxidants, effectively scavenging excessive oxygen free radicals within cells and safeguarding the integrity of the intestine against oxidative damage [13]. Additionally, the structure and functionality of the intestinal microbiota indirectly mirror the state of host intestinal health in the intestinal microecosystem of fish [14]. In line with advancements in molecular biology techniques, the application of high-throughput sequencing of 16S rRNA and other cutting-edge technologies has gained momentum in assessing the intestinal microbiota of fish. This shift in focus from solely analyzing microbial community structure to investigating microbial community function has paved the way for a more comprehensive understanding [15,16]. However, the intestinal microbiota is not static and can vary according to environment, diet, host behavior, differentiation stage, and genotype. Prior studies have demonstrated significant alterations in enzyme activity and microbial diversity within the intestines of *Sparus aurata* [17] and *Oncorhynchus mykiss* [18] under varying stocking densities.

The aquaponics system represents an intricately integrated aquaculture model that harmoniously amalgamates recirculating aquaculture systems with soil-less cultivation techniques, thereby embracing a holistic and interdisciplinary approach [19]. Prior studies have unveiled the profound merits of aquaponics in augmenting water quality and bolstering aquaculture productivity [20,21]. However, extant scholarship predominantly fixates on optimizing the overall system dynamics, encompassing topics such as water quality, vegetable assortment, quality, and yield, while regrettably overlooking the meticulous examination of fish intestinal microbiota within this paradigm. Furthermore, stocking density assumes a pivotal role in shaping the productivity of aquaculture water and engenders a multifaceted mechanism, involving intricate interplays among various contextual factors contingent upon divergent circumstances [22]. Hence, this study endeavors to meticulously evaluate the ramifications of distinct stocking densities on the intestinal tissue morphology, digestive enzyme activity, antioxidant enzyme activity, and microbial community of *M. salmoides* inhabiting an industrial aquaponics system. By unearthing the underlying mechanisms underscoring the influence of stocking density on the intestinal health of *M. salmoides*, this work strives to furnish a solid theoretical foundation for expediting the adoption of salubrious pisciculture technologies.

2. Materials and Methods

2.1. Experimental Material

The feed was procured from Chongqing Haida Feed Co., Ltd. (particle size: 2.0, crude protein $\geq$ 49.0%, crude fiber $\leq$ 3.5%, crude ash $\leq$ 18.0%, calcium: 0.80–4.0%, total phosphorus $\geq$ 1.2%, crude fat $\geq$ 5.0%, lysine $\geq$ 3.0%, moisture $\leq$ 10.0%). Juvenile *M. salmoides* bass were acquired from a piscicultural facility in Guangdong and nurtured at Liangping Shugu Farm in Chongqing City until they attained an average body length of 10.13 $\pm$ 0.42 cm and an average body weight of 27.59 $\pm$ 3.34 g. Preceding the commencement of the experiment, the juvenile *M. salmoides* bass underwent a fortnight of transient rearing within a circular recirculating aquaculture tank encompassing a volumetric capacity of 100 m^3 (D $\times$ H: 8 $\times$ 2 m). Concurrently, lettuce cultivation was initiated within the proximate vegetable area interconnected with the piscine reservoir, thereby establishing an integrated aquaponics system. The corresponding planting area of the whole system was 300 m^2 and the planting density was 18 trees/m^2. Throughout the temporary rearing phase, optimal aeration techniques were employed to ensure sufficient oxygenation within all tanks, while vital physicochemical parameters such as dissolved oxygen levels, water temperature, and pH were meticulously assessed daily. The concentration of dissolved oxygen within the experimental water was meticulously maintained above 8 mg/L, whereas the water temperature was strictly upheld between 23–28 °C, and the pH was meticulously regulated within the range of 7.0–8.0. Regular volumetric water replacements were meticulously executed, constituting 1% of the entire culture tank volume. The system mainly consisted of six tanks, microfilters, fluidized beds, ultraviolet lamps, and other auxiliary facilities. After treatment through the filters, fluidized beds, and UV lamps, the water flowed to the lettuce growing area and then returned to the fish pond.

2.2. Experimental Methods

The experimentation was carried out at the Aquaponics AI Factory situated in Liangping District, Chongqing City. The experimental setup encompassed two meticulously designed culture tanks, wherein the juvenile *M. salmoides* bass were judiciously stocked at densities of 160 fish/m^3 (HSD) and 120 fish/m^3 (LSD), respectively. Three replicates were set for each treatment group. The duration of the culture phase spanned a comprehensive 40-day timeframe, wherein the piscine inhabitants received a carefully calculated daily dietary intake equivalent to 2% of their body mass. Fish weight was measured every 7 days and the 2% dietary intake re-calculated. Feeding regimens were meticulously adhered to, with nutrient provision occurring twice daily at precise timings of 9:00 a.m. and 18:00 p.m. The morning feed comprised 1% of the overall ration, followed by an equivalent portion being tendered during the evening hours. Continuous aeration with liquid oxygen was faithfully maintained throughout the entire experimental period to optimize the oxygen saturation levels within the culture tanks. An orchestrated water replacement procedure was diligently executed once, entailing the meticulous substitution of 1% of the total volume of water. The requisite parameters pertaining to water quality were scrupulously gauged and judiciously upheld in strict accordance with the standards established during the temporary rearing phase, commencing 1 h subsequent to each feeding event.

Upon the culmination of the aquaculture undertaking, a meticulous sampling procedure was undertaken. The harvested juvenile *M. salmoides* specimens were rendered inert through the administration of 100 mg/L MS-222, subsequently transferred onto an ice tray. To ensure the elimination of any extraneous impurities, the piscine subjects underwent a thorough cleansing process involving 70% ethanol and sterile water solutions. In an environment characterized by sterility, the intact intestinal tracts of each fish were delicately extracted. The procured intestinal tissue samples were then segregated into two discrete portions. The first component was suitably preserved in a solution comprising 4% paraformaldehyde, while the second fraction was carefully placed within a centrifuge tube and stored at an ultra-low temperature of -80 °C within a freezer facility. Furthermore, the contents of the intestines from three representative fish in each treatment group were amal-

gamated to generate a unified composite sample. Three parallel samples were collected for each respective treatment group and diligently preserved at −80 °C until such time that microbial sequencing analysis could be performed.

2.3. Growth Performance Analysis

Prior to the conclusion of the aquaculture trial, a 24 h fasting period was imposed. Eleven fish were randomly selected from each treatment group, and their body weight and length were measured to calculate morphometric indices. The formulas for calculation are as follows:

$$\text{Weight gain ratio (WGR, \%)} = (\text{Final average body weight} - \text{Initial average body weight})/\text{Initial average body weight} \times 100\%$$

$$\text{Specific growth rate (SGR, \%)} = (\ln(\text{Final average body weight}) - \ln(\text{Initial average body weight}))/\text{Experimental duration} \times 100\%$$

$$\text{Condition factor (CF, \%)} = 100 \times \text{Body weight}/(\text{Body length})^3$$

2.4. Histological Analysis

The anterior intestine, middle intestine, and posterior intestine (obtained by dividing the intestine into three equal parts) of the juvenile *M. salmoides* were processed into paraffin sections for HE staining. The sections were observed using an Olympus BX 51 (Olympus, Tokyo, Japan) microscope, and five fields of view were selected for each slide. The measurements of villus height (hS), muscular layer thickness (tM), villus width (wS), and crypt depth (dC) were performed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

2.5. Determination of Enzyme Activity

In this study, the activity of intestinal amylase, protease, lipase, superoxide dismutase (SOD), glutathione-S-transferase (GST), glutathione peroxidase (GPx), and glutathione (GSH), and the content of glutathione (GSH) and malondialdehyde (MDA), were determined with kits from Nanjing Constructive Biotechnology Institute (Nanjing, China).

2.6. Extraction of DNA from the Sample

The DNA of microbial contents in the gastrointestinal tract was extracted using the Stool DNA Kit (OMEGA, Norcross, GA, USA). The DNA extraction steps for all samples followed the instructions provided with the Stool DNA Kit, and the quality of the extracted genomic DNA was evaluated using 1% agarose gel electrophoresis.

2.7. High-Throughput Sequencing Analysis

The specific primers 338F/806R containing a barcode were designed for the V3 + V4 region of the bacterial 16S rRNA gene. The primer sequences were 338F:5′–ACTCCTACGGG AGGCAGCA–3′ and 806R:5′–GGAC-TACHVGGGTWTCTAAT–3′. PCR amplification was performed using an rTaqDNA Polymerase-20 μL reaction system, 10 × Buffer 2 μL, 2.5 mmoL·L^{-1} DNTPS 2 μL, primer 338F/806R 0.8 μL, Taq enzyme 0.2 μL, BSA 0.2 μL, template DNA 10 ng, and ddH_2O supplemented to 20 μL. The PCR conditions were denaturated at 95 °C for 3 min, 27 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s. The final extension was 72 °C for 10 min (PCR instrument ABI Gene-Amp 9700, Thermo Fisher Scientific, Waltham, MA, USA).

The PCR products were subjected to 2% agarose gel electrophoresis, resulting in clear bands with the appropriate fragment size (430 bp). The gel-extracted PCR products were assessed using the AxyPrepDNA Gel Recovery Kit (Axygen, Saint Louis, MO, USA), followed by ligation of "Y" adapters after Tris-HCl elution. Magnetic bead selection was employed to remove adapter-dimer fragments. To enrich the PCR products, a standard enrichment PCR was conducted with 1 ng of PCR product for each sample, and subsequently denatured with

a 0.1 M NaOH solution to obtain single-stranded DNA fragments. The sequencing library was constructed, and high-throughput sequencing was performed on the 16S-338F-806R Miseq platform (Shanghai Meiji Biotechnology Co., Ltd., Shanghai, China).

2.8. Data Analysis

The experimental data are presented as mean ± standard error (mean ± SE). One-way analysis of variance (ANOVA) was performed using SPSS 23 (IBM, USA) on the aforementioned data, followed by Tukey's honestly significant difference (HSD) test for multiple comparisons. The significance level (p-value) was set at 0.05, indicating a significant difference when $p \leq 0.05$, and a highly significant difference when $p \leq 0.01$.

3. Results and Analysis

3.1. Growth and Intestinal Morphology of Juvenile M. salmoides Cultured at Different Densities

At the end of the experiment, the average body weight, weight gain rate, and specific growth rate of the HSD group were significantly higher than those of the LSD group ($p \leq 0.05$). However, there was no significant difference in condition factor ($p > 0.05$) among the groups (Table 1).

Table 1. Effects of different densities on growth indices of juvenile *M. salmoides*.

Group	IBW (g)	FBW (g)	WGR (%)	SGR (%/Day)	CF (g/cm^3)
HSD	26.38 ± 3.48	93.50 ± 12.68 *	274 ± 70 *	3.15 ± 0.51 *	2.77 ± 0.10
LSD	29.03 ± 3.21	83.89 ± 12.10	193 ± 39	2.62 ± 0.34	2.81 ± 0.17

Note: * significant significant differences from LSD group.

After the completion of the aquaculture experiment, morphological analysis was conducted on the intestinal tract of juvenile *M. salmoides* (Figure 1). The structure of the intestine appeared relatively intact, with well-organized intestinal villi and a smooth mucosal surface. Measurements were taken for villus height (Vh), villus width (Vw), crypt depth (Cd), and muscle layer thickness (Mt) in different sections of the intestine, and the results are presented in Table 2. For the anterior intestine, the villus width of HSD groups was significantly higher than that of LSD groups ($p \leq 0.05$). In the middle intestine, both villus height and muscle layer thickness of HSD groups were significantly higher than those of LSD groups ($p \leq 0.01$). As for the posterior intestine, the villus height of HSD groups was significantly higher than that of LSD groups ($p \leq 0.01$). There were no significant differences in crypt depth among the treatment groups and in the anterior, middle, and posterior sections of the intestine ($p > 0.05$).

Table 2. Measurements of intestinal tract indexes of juvenile *M. salmoides* in different density treatment groups.

Group	Villus Height (μm)	Villus Width (μm)	Crypt Depth (μm)	Muscular Thickness (μm)
Foregut HSD	415.98 ± 8.37	83.12 ± 4.54 *	28.93 ± 1.49	97.92 ± 8.43
Foregut LSD	471.33 ± 36.7	65.26 ± 4.45	25.03 ± 1.45	80.85 ± 2.61
Midgut HSD	557.88 ± 19.44 **	97.07 ± 14.34	32.91 ± 2.35	168.28 ± 6.75 **
Midgut LSD	428.3 ± 16.47	88.86 ± 5.49	28.73 ± 1.34	102.3 ± 6.62
Hindgut HSD	668.52 ± 23.84 **	84.07 ± 10.17	28.13 ± 1.14	112.56 ± 12.57
Hindgut LSD	233.1 ± 31.64	74.22 ± 9.83	27.57 ± 1.46	102.06 ± 9.7

Note: * significant and ** extremely significant differences from LSD group.

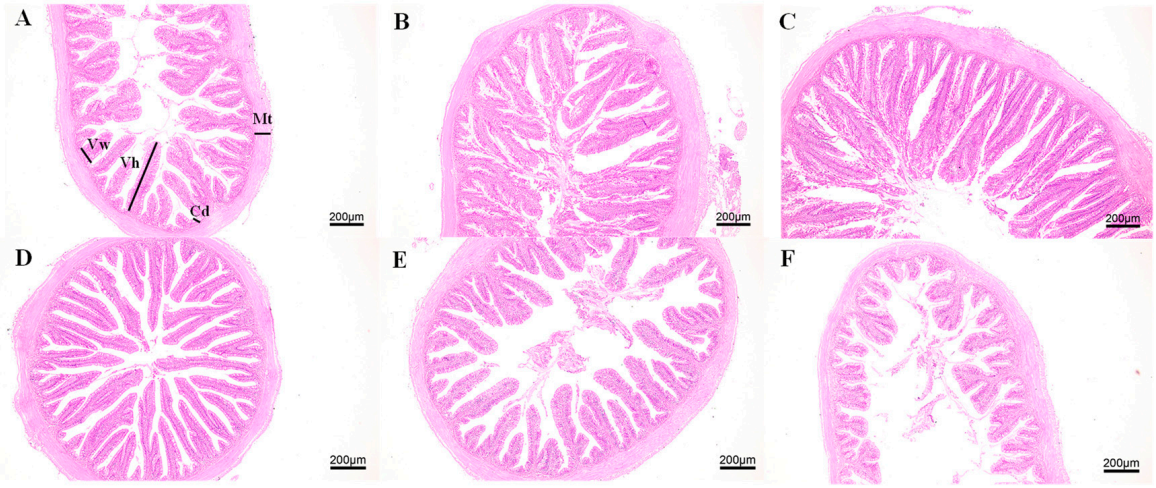

Figure 1. Microscopic structure of intestinal tract of juvenile *M. salmoides* bass in HSD and LSD groups (40×): (**A**–**C**) show the foregut, midgut, and hindgut of juvenile *M. salmoides* in the HSD group, respectively; (**D**–**F**) show the foregut, midgut, and hindgut of the intestinal tract of juvenile *M. salmoides* in the LSD group, respectively. Vh, villus height; Vw, villi width; Cd, crypt depth; Mt, muscular thickness.

3.2. Changes in Enzyme Activities in Intestinal Tissues of Juvenile M. salmoides Cultured at Different Densities

After the completion of the aquaculture experiment, significant changes were observed in the digestive enzyme activities of juvenile *M. salmoides*. Specifically, the protease activity ($p \leq 0.05$) (Figure 2A) and lipase activity ($p \leq 0.01$) (Figure 2C) of the LSD group were significantly higher than those of the HSD group, while the amylase activity was significantly lower in the LSD group compared to the HSD group ($p \leq 0.05$) (Figure 2B).

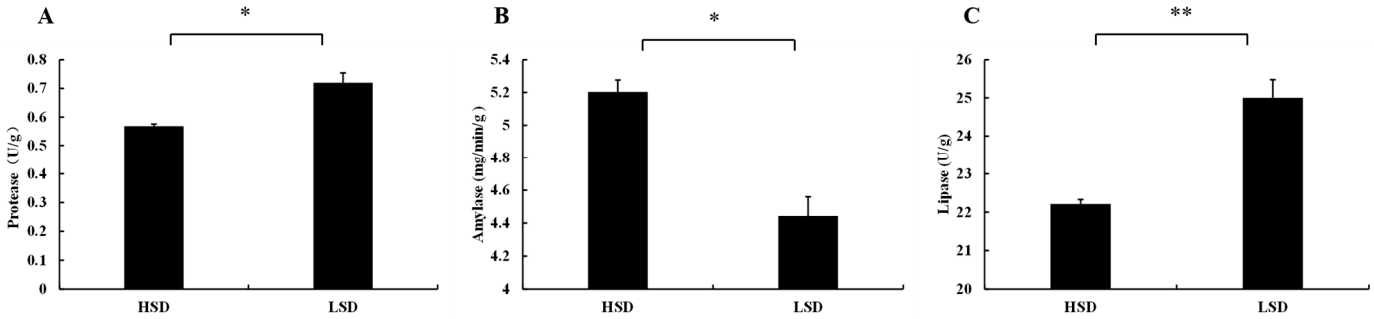

Figure 2. Effects of different culture densities on digestive enzyme activities in intestinal tissues of juvenile *M. salmoides*. Note: * significant and ** extremely significant differences from the LSD group. (**A**) protease (U/g); (**B**) amylase (mg/min/g); (**C**) lipase (U/g).

After the conclusion of the aquaculture experiment, significant changes were observed in the antioxidant enzyme activities of the intestinal tract in juvenile *M. salmoides*. Specifically, the SOD activity ($p \leq 0.01$) (Figure 3A), GST activity ($p \leq 0.01$) (Figure 3B), and GPx activity ($p \leq 0.05$) (Figure 3C) were significantly higher in the HSD group compared to the LSD group. Moreover, the GSH content in the HSD group was significantly higher than that in the LSD group ($p \leq 0.05$) (Figure 3D). However, there were no significant differences in the MDA content between the LSD group and the HSD group ($p > 0.05$) (Figure 3E).

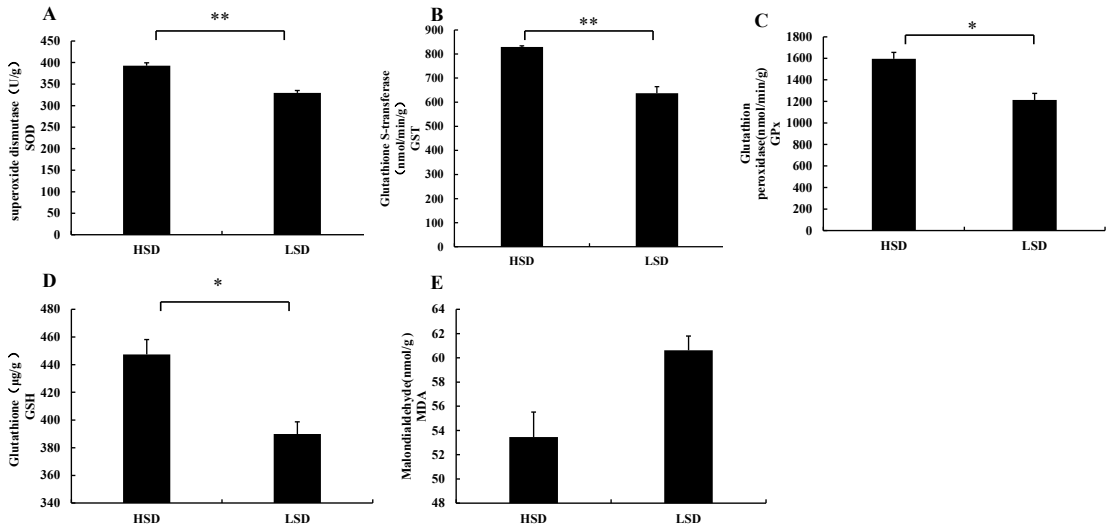

Figure 3. Effects of different culture densities on antioxidant enzyme activities in intestinal tissues of juvenile *M. salmoides*. (**A**) SOD (U/g); (**B**) GST (nmol/min/g); (**C**) GPx (nmol/min/g); (**D**) GSH (μg/g); (**E**) MDA. Note: * significant and ** extremely significant differences from the LSD group.

3.3. Intestinal Microbial Structure of Juvenile M. salmoides at Different Breeding Densities

Statistical analysis was conducted on the Alpha diversity of intestinal samples from juvenile *M. salmoides*. The Chao1, Good's coverage, and Simpson indices obtained in this experiment are presented in the figure below, aiming to explore richness, diversity, and coverage characterization. The results revealed that the community coverage of each sample exceeded 99.97%, indicating a low probability of unsequenced samples. There were no significant differences in the Chao1, Coverage, and Simpson indices between each sample of the two groups of fish ($p > 0.05$) (Figure 4).

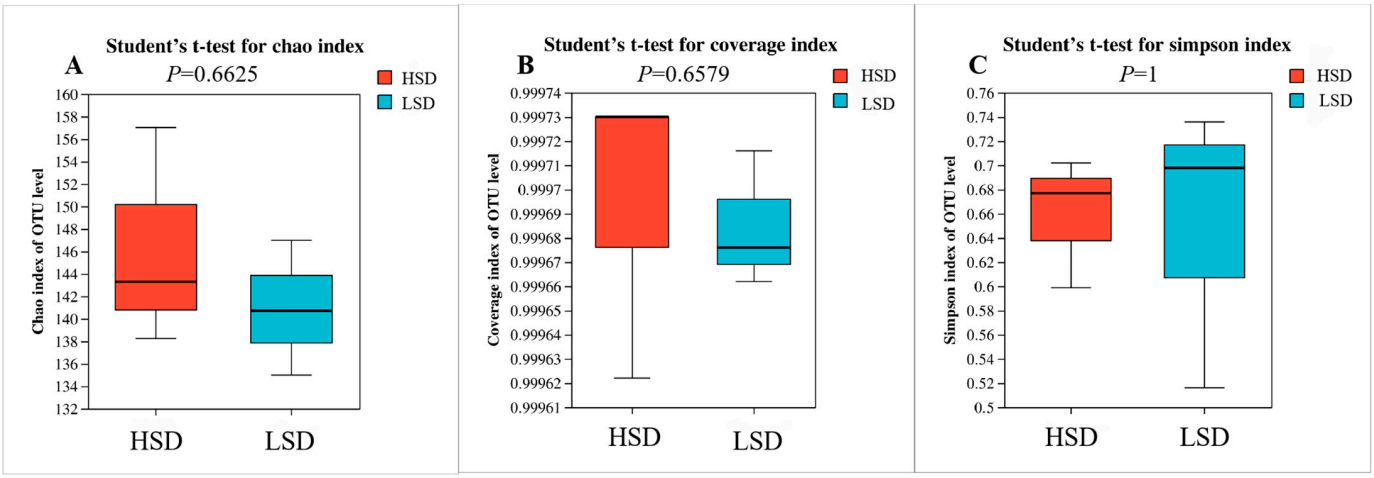

Figure 4. Alpha diversity analysis of intestinal microorganisms in juvenile *M. salmoides*. (**A**) Chao index of OTU level; (**B**) coverage index of OTU level; (**C**) Simpson index of OTU level.

In the HSD and LSD treatment groups, a total of 11 phyla were detected in the intestinal tract of juvenile *M. salmoides*. These phyla mainly consisted of Firmicutes, Proteobacteria, Cyanobacteria, Actinobacteriota, and Fusobacteriota, although their proportions varied (Figure 5A). In the LSD group, Firmicutes (84.04%), Proteobacteria (12.44%), and Cyanobacteria (3.28%) were predominant, accounting for 99.76% of the total abundance. Similarly, in the HSD group, Firmicutes (85.80%), Proteobacteria (12.42%), and Cyanobacteria (1.64%) were the major phyla, representing 99.86% of the total abundance. Firmicutes were the

dominant phylum in all groups, but there were no significant differences between the groups ($p > 0.05$).

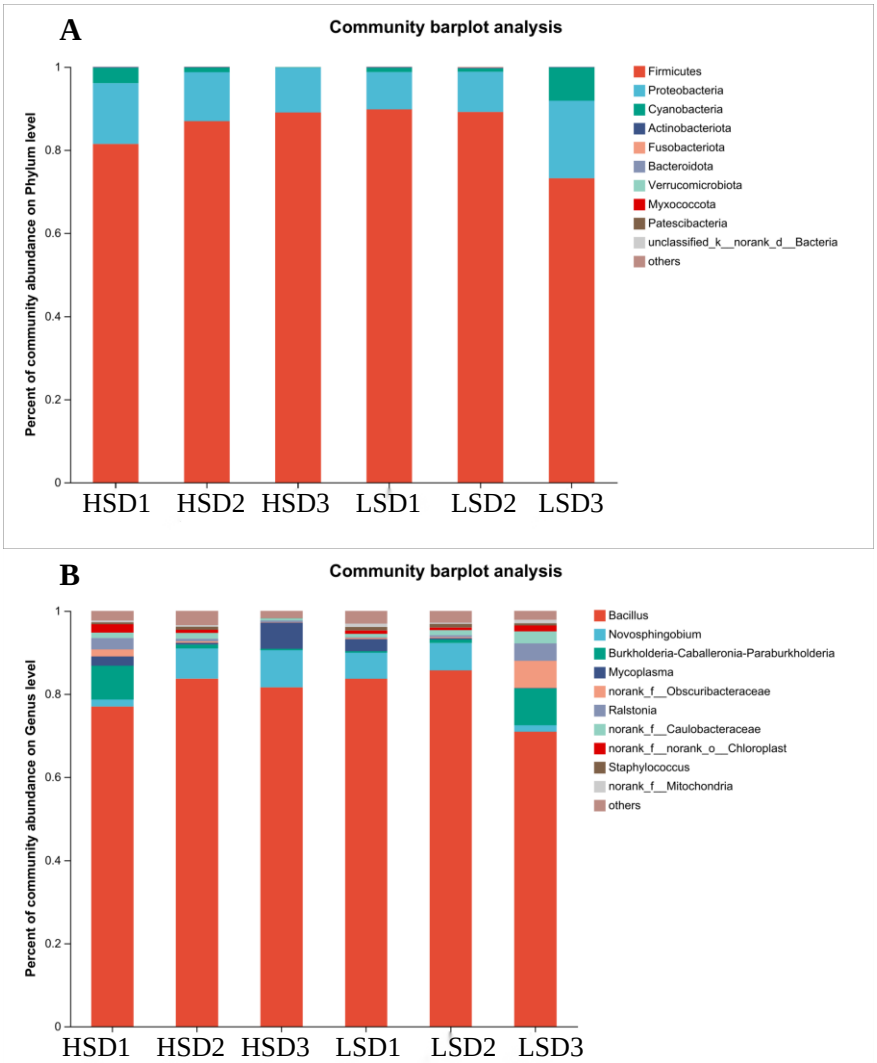

Figure 5. Relative abundance of intestinal bacteria at phylum level and genus level in juvenile *M. salmoides* from different density treatment groups. Three numbers in the same group represent three replicate treatments. (**A**) Percent of community abundance on Phylum level; (**B**) Percent of community abundance on Genus level.

In the stacked bar plot of bacterial genus-level relative abundance, a total of 239 genera were identified in the intestinal tract of juvenile *M. salmoides*, with the top 10 genera accounting for over 96% of the abundance in each group. In both density groups, the dominant genera in terms of abundance were *Bacillus*, *Novosphingobium*, and *Burkholderia*, in that order (Figure 5B). In the LSD group, the dominant genera were *Bacillus* (79.83%), *Novosphingobium* (4.77%), *Burkholderia* (3.42%), and *Mycoplasma* (1.07%), together representing 89.09% of the total abundance. In the HSD group, the dominant genera were *Bacillus* (80.58%), *Novosphingobium* (5.96%), *Burkholderia* (3.13%), and *Mycoplasma* (3.00%), accounting for 92.67% of the total abundance. *Bacillus* was the overwhelmingly dominant genus in all groups, but there were no significant differences between the groups ($p > 0.05$).

The microbial communities in the intestinal tract of juvenile *M. salmoides* in the HSD and LSD treatment groups were subjected to COG functional prediction and classification analysis using the PICRUST tool. The results showed that both groups had 22 functional clusters, with relatively higher abundance in the categories of amino acid transport and

metabolism, transcription, and inorganic ion transport and metabolism. There were no significant differences in functional classification between the different density treatment groups ($p > 0.05$) (Figure 6).

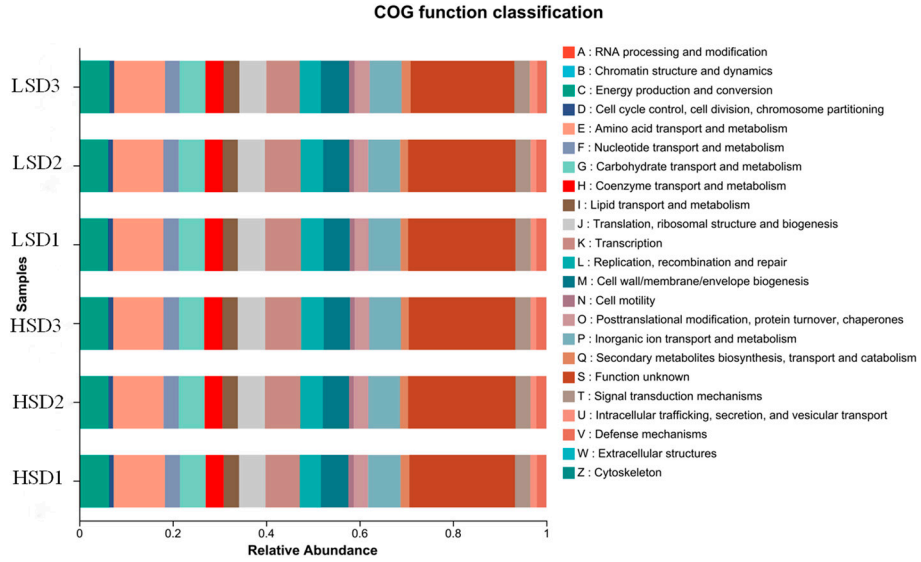

Figure 6. Prediction of intestinal COG function in juvenile *M. salmoides*. Three numbers in the same group represent three replicate treatments.

4. Discussion

4.1. Effects of Culture Density on Growth and Intestinal Morphology of Juvenile M. salmoides

In the realm of aquaculture, stocking density has long been recognized as a pivotal factor exerting influence over growth dynamics [23]. Scientific studies from the past have elucidated the profound impact of high stocking density on some species, instigating a cascade of physiological impairments and, in severe cases, mortality [24,25]. Conversely, reduced stocking density fails to optimize the utilization of aquacultural waters, leading to diminished production yields. Moreover, it curtails interindividual interactions among fish, thereby compromising their growth potential and physiological capacities [26]. Hence, each fish species possesses an optimal stocking density range capable of eliciting peak growth performance [27]. The findings of this empirical endeavor elucidate a conspicuous disparity between the HSD and LSD experimental groups in terms of average body weight, weight gain, and specific growth rate of juvenile *M. salmoides*. The results of this study indicated that the average body weight, weight gain, and specific growth rate of juvenile *M. salmoides* in the HSD group were significantly higher than those in the LSD group. It suggests that, in this particular mode, the stocking density in the HSD group can enhance the growth performance of juvenile *M. salmoides*. This observation bears striking resemblance to the outcomes documented by Zhu et al. [26], who expounded upon the impact of density variations on the growth of juvenile *Pseudobagrus ussuriensis*. In summary, judiciously augmenting the stocking density of juvenile *M. salmoides* holds the potential to ameliorate both production yield and aquacultural efficiency.

The intestinal tract of fish is commonly partitioned into three distinct segments: the foregut, midgut, and hindgut [28]. Functionally, the foregut primarily assumes responsibility for digestion and absorption, whereas the midgut and hindgut play a pivotal role in immune-related processes [29]. Notably, the digestive and absorptive efficiency of the fish intestine is intricately linked to the condition of its mucosal surface structure [30]. Morphological parameters such as villus height, villus width, muscular layer thickness, and crypt depth are frequently employed as metrics to assess intestinal development, health status, and digestive absorptive capacity [31,32]. Heightened villus height and width signify an increased presence of epithelial cells, which, in turn, indicates a heightened nutrient absorption potential within the intestine. Conversely, reduced villus dimensions

point toward an inverse scenario [33]. In the present study, notable disparities in villus height and width within the foregut, midgut, and hindgut of juvenile *M. salmoides* were observed between the HSD and LSD experimental groups. This suggests that rearing juvenile *M. salmoides* at a higher density in the HSD group can increase the absorptive surface area of their intestines, enhance their digestive and absorptive capacity, and improve intestinal structure, thereby maximizing their growth potential. Importantly, the thickening of the intestinal muscular layer expedites peristaltic contractions, consequently amplifying intestinal absorption rates [34]. The substantially augmented muscular layer thickness observed within the foregut of the HSD group, relative to the LSD group, further corroborates this notion. In sum, the divergences in intestinal morphology provide tangible evidence of variances in the intestinal architecture of juvenile *M. salmoides* across distinct stocking densities. Fish specimens reared within the HSD group exhibit heightened digestive and absorptive capacities, alongside a healthier intestinal milieu.

4.2. Effects of Different Culture Densities on Enzyme Activities in Intestinal Tissues of Juvenile M. salmoides

The level of digestive enzyme activity serves as an indicator of the alimentary capability and absorptive prowess of piscine organisms with respect to nutrient assimilation [35]. In addition to environmental factors such as temperature [36], pH [37], salinity [38], and dissolved oxygen [39], stocking density also has a significant impact on digestive enzyme activity [40]. Previous studies have shown that cultured fish exhibit optimal digestive physiological status and higher activity of digestive enzymes in their tissues within an appropriate stocking density [41]. However, when the density exceeds the carrying capacity of the culture system, the digestive enzyme activity in the fish is actually inhibited. Exemplifying this, augmentation in stocking density prompts a concomitant decrement in the activities of five digestive enzymes residing within the gastric and intestinal compartments of *Larimichthys crocea* [42]. Ergo, the extent of digestive enzyme activity within cultured fish directly corresponds to stocking density. In this study, the protease and lipase activities in the juvenile *M. salmoides* were significantly higher in the LSD group than in the HSD group. This discrepancy intimates that diminished stocking density engenders heightened digestive enzyme activity within juvenile *M. salmoides*, thereby concomitantly propelling the process of nutrient digestion and absorption enacted by the enteral tissues. This finding aligns with the observations imparted by Ezhilmathi et al., who expounded upon the ramifications of stocking density on digestive enzyme activity within *Lates calcarifer* [24]. However, the amylase activity in the HSD group of juvenile *M. salmoides* was significantly higher than that in the LSD group. This divergence likely stems from the adaptive capacity exhibited by the juvenile *M. salmoides* towards elevated stocking densities, whereby an augmentation in amylase activity facilitates efficacious digestion and utilization of residual sustenance, consequently sharpening their survival aptitude within the milieu. This parallels the conspicuous surge in amylase activity documented in *Cyprinus carpio* subsequent to bouts of nutrient deprivation [43].

The intestinal tract of fish serves not only as a site for digestion but also actively participates in immune responses, metabolic processes, and various stress reactions [44]. Extensive research has revealed that high stocking density induces stress responses in fish intestinal cells, leading to an excessive generation of reactive oxygen species (ROS) that can inflict damage upon cells, tissues, and other vital components within the fish [45]. However, throughout the course of evolution, organisms have evolved a sophisticated antioxidant defense system to effectively eliminate superfluous free radicals and safeguard cellular integrity [46]. This antioxidant system primarily comprises enzymatic and non-enzymatic components, with the enzymatic system encompassing key enzymes such as superoxide dismutase, glutathione transferase, and glutathione peroxidase, all instrumental in the elimination of ROS [47,48]. On the other hand, the non-enzymatic system chiefly involves reduced glutathione, which serves as an adept scavenger of oxygen free radicals and acts as a detoxifying agent against electrophiles, thereby maintaining the equilibrium of

thiol-disulfide bonds and facilitating signal transduction processes [49]. Malondialdehyde (MDA) is the end product of lipid peroxidation, and its content combined with changes in various antioxidant enzymes can collectively reflect the oxidative stress encountered by fish [50]. Under conditions of high stocking density, free radicals directly attack polyunsaturated fatty acids within membranes, thus initiating lipid peroxidation reactions [51]. In this study, the activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione transferase (GST), as well as the content of reduced glutathione (GSH) within the intestinal tract of juvenile *M. salmoides*, were found to be significantly higher in the HSD group compared to the LSD group. There was no discernible disparity observed in malondialdehyde (MDA) levels, indicating that a stocking density of 160 fish/m^3 only increases the antioxidant enzyme activity in the intestinal tract of juvenile *M. salmoides*. However, through the upregulation of their endogenous antioxidant enzymes, juvenile *M. salmoides* can effectively eliminate excessive ROS and superoxide radicals, thereby circumventing oxidative damage. Consequently, based on the comprehensive alterations in digestive enzyme and antioxidant enzyme activities within the intestinal tract of juvenile *M. salmoides*, it can be inferred that the stocking density employed in the HSD group is relatively suitable during the juvenile stage.

4.3. Effects of Different Culture Densities on Intestinal Microorganisms in Juvenile M. salmoides

The intestinal microbiota in fish exhibits a vast assemblage of species and a substantial population [52]. It fulfills an indispensable role in the decomposition and synthesis of essential nutrients required by the host, augmentation of nutrient absorption within the intestinal milieu, production of digestive enzymes, and regulation of protein expression associated with metabolic processes [53]. Studies have demonstrated that stocking density exerts a significant influence on the intestinal microbiota of fish, thereby influencing their growth by modulating the activity of digestive enzymes and antioxidant enzymes [54]. The present study scrutinized the configuration of the intestinal microbial community in juvenile *M. salmoides* reared within an aquaponics system at distinct density levels. Alpha diversity indices divulged no noteworthy disparities in the diversity of bacterial communities among the various groups, which is consistent with the findings of Xu et al. on the impact of dissolved oxygen on the intestinal microbiota of *Salmo salar* [55]. Additionally, this study verified that the prevailing bacteria residing within the intestinal microbiota of juvenile *M. salmoides* primarily belong to the phyla Firmicutes and Proteobacteria [56]. Proteobacteria are known to be abundant in stable (healthy) gut microbiota [57], while Firmicutes, particularly the *Bacillus* genus, are major bacteria involved in the metabolism of food residues [58]. These bacteria foster digestion and enhance nutrient assimilation efficacy while simultaneously acting as pivotal microorganisms engaged in disease prevention and control via the production of antimicrobial proteins or enzymes eliciting host resistance against diseases [59]. However, a significantly heightened abundance of *Chlamydia* was observed in the HSD group relative to the LSD group. *Chlamydia*, a member of the Chlamydiae phylum, represents one of the smallest self-replicating free-living microorganisms known to date [60]. It is also a potential pathogenic bacterium that can cause intestinal-related diseases such as enteritis [61]. Based on these findings, it is speculated that elevated stocking density may augment the abundance of potentially pathogenic bacteria, consequently instigating intestinal damage, which aligns with prior research outcomes concerning *M. salmoides* [62]. Thus, it is advisable to exercise heightened vigilance pertaining to pathogenic microorganisms such as Chlamydia during the farming process to preclude disease outbreaks and mitigate fish mortality.

The intestinal microbiota of organisms possesses the capacity to ferment dietary amino acids, resulting in the production of diverse metabolites [63]. Through the modulation of gut microbiota, the host can derive advantageous outcomes by fine-tuning its own metabolic processes, thereby enabling superior adaptation to novel nutritional and environmental exigencies [64]. Hence, delving into the functional transformations occurring within the gut microbiota yields a more comprehensive comprehension of the profound

impact imposed upon the host by perturbations in its intestinal microbial milieu. Functional predictions have unearthed that the intestinal microbial community of juvenile *M. salmoides* primarily engages in pivotal activities encompassing amino acid transportation and metabolism, transcription, as well as inorganic ion transportation and metabolism. These functions are beneficial for the utilization of nutrients by the fish's body and the proper functioning of cellular activities, ultimately promoting the fish's adaptability to the environment. Nonetheless, it is imperative to acknowledge that prevailing research outcomes predominantly hinge upon sequencing data and functional pathway prognostications, with restricted validation data at hand. Thus, further investigations are warranted to unravel the underlying mechanisms at play.

4.4. Comparison of the Dominant Intestinal Microflora of M. salmoides under Different Factors

The composition of the intestinal microbiota in fish is shaped by the intricate interplay between host physiological traits and external environmental factors [65]. Fish reside within a dynamic ecological system, wherein fluctuations in endogenous or exogenous elements wield the potential to impact both the structure and diversity of their intestinal microbiota [66]. Comprising archaea, bacteria, protists, fungi, and viruses [49], the fish intestinal microbiota is predominantly characterized by the bacterial contingent [67]. Notably, within the realm of bacteria, notable representatives from the phyla Proteobacteria, Firmicutes, and Bacteroidetes have been identified across diverse fish species, constituting a substantial proportion—upwards of 90%—of the investigated fish intestinal microbiota. This observation not only signifies the putative significance of these bacterial cohorts in shaping fish intestinal function but also underscores their pivotal contributions [68]. Detailed insights into the factors influencing the intestinal microbiota of *M. salmoides* are presented in Table 3, underscoring the preponderance of Firmicutes, Proteobacteria, and Bacteroidetes at the phylum level. At the genus level, dominant bacterial taxa encompass *Mycoplasma*, *Leptothrix*, and *Aeromonas*. Moreover, distinctions in the structure and diversity of the gut microbiota in *M. salmoides* may be ascribed, in part, to the employed farming methodologies, including pond culture, laboratory recirculating water culture, pond recirculating water culture, factory recirculating water culture, and cage culture. Intriguingly, feed composition incorporating nutritional constituents and additives, the aquatic milieu encompassing dissolved oxygen levels and environmental pollutants, as well as the developmental stage of the host individual, all emerge as influential determinants in orchestrating the establishment of intestinal microbiota in juvenile *M. salmoides*. Ultimately, these factors culminate in the composition of a relatively stable microbiota within adult individuals. In conclusion, to ensure a stable intestinal microbiota structure in *M. salmoides* during cultivation, it is necessary to adjust the nutritional strategies according to different environmental conditions and growth stages, aiming to achieve efficient and healthy growth.

Table 3. The dominant intestinal microbiota of *M. salmoides* under different factors.

No.	Experimental Site	Influence Factor	Dominant Flora (Phylum Level)	Dominant Flora (Genus Level)	References
1	Indoor recirculating aquaculture system/laboratory	Fish protein hydrolysates	Fusobateriota, Firmicutes, Cyanobacteria	*Cetobaterium*, *Staphylococcus*, *Kocuria*	[69]
2	Tanks/laboratory	Chinese herbal medicine	Proteobacteria, Firmicutes, Fusobacteria		[70]
3	Concrete tanks	Methionine hydroxy analogue	Firmicutes, Bacteroidetes, Proteobacteria	*Clostridiales*, *Faecalibacterium*, *Bifidobacterium*	[71]
4	The blue circle cylinder/laboratory	Yeast culture	Proteobacteria, Firmicutes, Actinobacteria	*Plesiomonas*, *Stenotrophomonas*	[14]
5	Recirculating aquaculture systems/laboratory	Enzymatic hydrolysis Soybean meal and soybean meal	Firmicutes, Fusobacteria, Proteobacteria	*Clostridium*, *Peptostreptococcaceae*, *Cetobacterium*	[72]
6	Glass fiber tank/laboratory	Lysophospholipids	Fusobacteria, Proteobacteria, Tenericutes	*Mycoplasma*, *Cetobacterium*, *Acinetobacter*	[73]

Table 3. *Cont.*

No.	Experimental Site	Influence Factor	Dominant Flora (Phylum Level)	Dominant Flora (Genus Level)	References
7	Glass fiber tank/laboratory	Alpha-lipoic acid	Fusobacteria, Proteobacteria, Firmicutes	*Cetobacterium*, *Lactobacillus*, *Lelliottia*	[73]
8	Aquarium/laboratory	Sodium butyrate	Cyanobacteria, Firmicutes, Tenericutes	*Mycoplasma*, *Fictibacillus*, *Lactobacillus*	[74]
9	Temperature-controlled circulating aquaculture system/laboratory	Fermented silkworm pupae meal	Proteobacteria, Actinobacteria, Firmicutes		[75]
10	Net cage	Fermented soybean meal	Tenericutes, Proteobacteria, Fusobacteria	*Mycoplama*, *Plesiomonas*, *Cetobacterium*	[76]
11	Aquarium/laboratory	Bacillus subtilis	Proteobacteria, Fusobacteria, Firmicutes		[77]
12	Indoor recirculating aquaculture system	The probiotic Bacillus sp		*Mycoplasma*, *Plesiomonas*, *Cetobacterium*	[78]
13	Net cage	Enzymatically treated Artemisia annua L.	Firmicutes, Fusobacteriota, Proteobacteria		[79]
14	Aquarium/laboratory	Starch level	Proteobacteria, Actinobacteria, Firmicutes	*Brevundimonas*, *Microbacteriaceae*, *Ralstonia*	[80]
15	Indoor recirculating aquaculture system/laboratory	Enzymatic chicken pulp	Proteobacteria, Firmicutes, Bacteroidetes		[81]
16	Glass fiber tank/laboratory	β–glucan	Fusobacteria, Firmicutes, Proteobacteria		[82]
17	Net cage	Feed protein level and small peptide addition	Proteobacteria, Tenericutes, Firmicutes	*Mollicutes*, *Mycoplasma*, *Achromobacter*	[83]
18	Net cage	Vitamin D3	Proteobacteria, Tenericutes, Firmicutes	*Mycoplasma*, *Plesiomonas*, *Cetobacterium*	[83]
19	Pond	Different diets	Firmicutes, Proteobacteria, Actinobacteriota		[84]
20	Pond	Different Growth Stages	Proteobacteria, Fusobacteria, Firmicutes	*Cetobacterium*, *Pseudomonas*, *Mycoplasma*	[85]
21	Pond	Different weaning Stages	Firmicutes, Tenericutes, Fusobacteria		[86]
22	Constant temperature recirculating aquaculture system	Different growth rates	Proteobacteria, Firmicutes		[11]
23	In-pond Raceway Aquaculture	Still water culture	Proteobacteria, Firmicutes, Cyanobacteria	*Acinetobacter*, *Delfti*, *Catenibacterium*	[87]
24	In-pond Raceway Aquaculture	Flowing water culture	Firmicutes, Proteobacteria, Acfinobacteria	*Mollicutes*, *Peptostreptococcaceae*, *Propionibacterium*	[87]
25	Pond	Natural culture	Fusobacteria, Proteobacteria	*Cetobacterium*, *Aeromonas*, *Fusobacterium*	[88]
26	Indoor tank/laboratory	Dissolved oxygen	Proteobacteria, Spirochaetes, Fusobacteria		[62]
27	Indoor recirculating aquaculture system/laboratory	Nanoplastics and Cd	Firmicutes, Tenericutes, Proteobacteria	*Mycoplasma*, *Aurantimicrobium*, *Dubosiella*	[89]

Note: the advantage bacterium group for the first three classes available in the literature; literature data were obtained from CNKI and Web of Science.

5. Conclusions

This study presents the inaugural exposition of the repercussions of divergent population densities upon intestinal tissue morphology, digestive enzyme activity, antioxidant enzyme activity, and microbial community in juvenile *M. salmoides*. Our findings elucidate that fish density within HSD groups engenders an augmented weight gain rate, and specific growth rate, as well as heightened dimensions of intestinal villi, muscle layer thickness, and intestinal antioxidant enzyme activity in juvenile *M. salmoides*. Such advancements in intestinal architecture concomitantly augment the digestive and absorptive prowess of the piscine subjects. Furthermore, Firmicutes and Proteobacteria constitute the predominant bacterial phyla within the intestinal microbiota. Nevertheless, the heightened prevalence of *Mycoplasma* within HSD groups may precipitate pathogenic occurrences, necessitating due regulation of the aquatic milieu to attenuate the presence of such pathogenic microorganisms during the cultivation process. By summarizing the research on the influence of

various factors on the intestinal microbiota of *M. salmoides* in recent years, it is evident that the intestinal microbiota is closely related to different farming modes, feed composition, water environment, and developmental stage. Therefore, adjusting the nutritional strategy based on specific conditions is necessary. These research findings have important implications for the healthy cultivation of juvenile *M. salmoides* with respect to fish density.

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Abbreviations

Abbreviations	Full name in English
HSD	160 fish/m^3, high stocking density
LSD	120 fish/m^3, low stocking density
IBW	initial average body weight
FBW	final average body weight
WGR	weight gain ratio
SGR	specific growth rate
CF	condition factor
hS	villus height
tM	muscular layer thickness
wS	villus width
dC	crypt depth
SOD	superoxide dismutase
GST	glutathione-S-transferase
GPx	glutathione peroxidase
GSH	glutathione
MDA	malondialdehyde

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